EXHIBIT B

LCR-mediated, long-term tissue-specific gene expression within replicating epis mal plasmid vectors

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Running title: **Episomal vect rs and locus control regions**

Summary

Replicating episomal plasmid vectors (REVs) have great potential in providing long-term expression within a gene therapy context. However, the question of how to obtain tissue-specific expression in REVs has been poorly addressed. Locus control regions (LCRs) are transcriptional regulatory elements which possess a dominant chromatin remodelling capability conferring on a gene linked *in cis* full physiological levels of expression when integrated into the host cell genome. Using the human β-globin LCR (βLCR) as a model, we have assessed the ability of this class of control elements to provide high level, tissue-specific gene expression from within an REV. The human β-globin gene either alone or linked to the DNaseI hypersensitive (HS) sites that constitute the βLCR, were incorporated into an Epstein-Barr virus (EBV)-based plasmid vector and introduced into erythroid K562 and non-erythroid HeLa cells. Combinations of βLCR HS site elements synergise to provide high level, tissue-specific expression. Importantly, the βLCR was also able to negate transgene silencing which otherwise occurred upon prolonged periods of culture. Our results demonstrate that the inclusion of appropriate control elements to maintain gene expression from within REVs are a crucial feature in vector design and that LCRs can fulfil this vital role.

Key words: episomal vectors; EBV; locus control region.

Introduction

The use of replicating episomal vectors (REVs)[1] offers an attractive alternative to integrating retroviral[2,3] and adeno-associated viral (AAV)[4] vectors for producing long term gene expression within a gene therapy context. Firstly, REVs do not pose the same size limitations on the therapeutic transcription unit as do viral vectors, with inserts in excess of 300kb being a possibility[5]. Secondly, being episomal, REVs do not suffer from potential hazards associated with insertional mutagenesis that is an inherent problem with integrating viral vectors. Lastly, REVs are introduced into the target cells using non-viral delivery systems that can be produced more cheaply at scale than with viral vectors[6-8]

REVs based on viral origins of replication such as those from EBV[9], human papovavirus BK[10,11], BPV-1[12] and SV40[13] hold the greatest promise. Combinations of viral and mammalian chromosomal origins of replication have also been found to improve nuclear retention of the episomes[14,15].

It has been demonstrated that both non-replicating, transiently transfected plasmids[16,17] as well as REVs[16,18,19] assemble nucleosomes. Assembly on REVs is more organised and resembles native chromatin whereas nucleosomes on transient plasmids are less well ordered allowing greater access of transcription factors to target sequences[17]. These observations imply that transcriptional control elements with a potent chromatin remodelling capacity may be required for efficient long term tissue-specific gene expression from within REVs. One such class of elements are locus control regions (LCRs)[20]. LCRs are tissue-specific regulatory elements which are

able to overcome chromatin position effects and confer upon a gene linked *in cis*, site-of-integration, physiological levels of expression directly proportional to transgene copy number in mice[21]. LCRs are able to obstruct the spread of heterochromatin and prevent position effect variegation (PEV)[22]. This pattern of expression conferred by LCRs suggests that these elements possess a powerful chromatin remodelling capability and are able to establish and maintain a transcriptionally competent, open chromatin domain. Therefore, LCRs are seen as the most efficient means of achieving reproducible, physiological levels of gene expression from stably integrated transgenes.

The requirements for tight tissue-specific gene expression from within REVs at a sustained level to be of long-term therapeutic benefit, has to date been poorly addressed. We have therefore assessed the ability of LCRs to drive efficient, tissue-specific gene expression from within REVs using the human β-globin LCR (βLCR) as a model system. The βLCR consists of five elements spread over 16kb of DNA located 5' of the entire β-globin gene family and which are characterised by high sensitivity to digestion with DNaseI (Figure 1)[20]. Components of the βLCR have been incorporated into both retroviral and AAV vectors[23]. The βLCR is now seen as an essential part of transcription units to reproducibly generate sufficiently high levels of β-globin to be value in gene therapy for the haemoglobinopathies[23].

In this study we have assessed the expression of the human β-globin gene either alone or in combination with elements of the βLCR within a REV and show that long term, high levels of erythroid-specific expression can be conferred by this element. This demonstrates the potential utility of this system for gene therapy applications.

Results

Episomal replication and copy number of LCR/β-globin gene constructs in K562 and HeLa cells

The β-globin gene either alone or in combination with one or more of the DNaseI hypersensitive (HS) sites that constitute the βLCR were inserted into the EBV-based vector p220.2 (Figure 1)[9]. These constructs were then used to generate stable transfected pools of the human myelogenous leukaemia cell line K562. These cells constitutively express the embryonic ε- and foetal γ-globin but not the adult β-globin genes[24]. This allows the β-globin reporter on the REVs to be quantified without interference from the homologous endogenous genes. In addition, four key constructs (β, 2β, 3β and 432β) were introduced into human HeLa cells which acted as a non-erythroid control.

The episomal status of the βLCR/β-globin gene plasmid constructs was determined as follows. Total DNA from the transfected pools of K562 cells was digested with *Bam*HI and *Hind*III and Southern blotted. These restriction enzymes liberate the p220.2 vector backbone as an 8.9 kb fragment (Figure 1A) only if the constructs remain as independent episomes. These blots were probed using the plasmid pBluescript that possesses extensive homology to pBR322 region of p220.2. The results (Figure 1B), show a single band corresponding to the size of linearised p220.2 indicating that within the K562 cells, the βLCR/β-globin gene episomal constructs were present only as unintegrated episomal DNA. Similar results were obtained for constructs transfected into HeLa cells (data not shown).

One of the pools harbouring the βLCR HS3-β globin gene construct (3β) was used as a representative sample to confirm the presence of episomal plasmid DNA by fluorescence *in situ* hybridisation (FISH) analysis of metaphase spreads. Figure 1C shows a typical example of 10 microscopic fields that were studied. As K562 cells are trisomy 11^{24} the results clearly showed three pairs of signals corresponding to the β-globin loci on these chromosomes (Figure 1C, arrows). No other pairs of signals associated with sister chromatids were detected indicating the absence of integrated copies of the transfected 3β construct. However, a number of single focal signals were observed consistent with the presence of episomal copies of 3β.

In order to determine if the βLCR/β-globin/REV constructs replicated extrachromosomally in K562 and HeLa cells, a low molecular weight DNA fraction was prepared[25], digested with *Dpn*I and re-transformed into *E. coli*. Since human cells lack DNA adenine methylase activity, replicated REV DNA is insensitive to digestion with *Dpn*I. This allows prokaryotic, input plasmid DNA to be differentiated from that which has undergone replication within either K562 or HeLa cells. Restriction enzyme analysis of plasmid DNA prepared from 5-10 colonies obtained with DNA rescued from transfected K562 or HeLa cells was found to be identical to the original input plasmid DNA used in the transfection, indicating that these plasmid molecules had replicated without detectable rearrangements within the human cells (data not shown).

The average copy number of the βLCR/β-globin gene episomal constructs in both K562 and HeLa cells, was estimated by Southern blot analysis of *Eco*RI-digested,

total DNA from stable transfected pools and probed with a 920bp fragment spanning IVS-II of the human β-globin gene (see Materials and methods; data not shown). The K562 pools had a copy number between 3 and 5 except for the βLCR HS3 construct containing cells that showed an average copy number of 10 (Figure 2B). The stable transfected HeLa cell pools had an average copy number of 1-2, except for the βLCR HS3 (3β) construct which contained on average 5 copies (Figure 2C).

Expression of LCR/β-globin gene episomal constructs in erythroid K562 cells.

Expression of the βLCR/β-globin gene/REV constructs in erythroid K562 cells was assessed using an S1-nuclease protection assay employing end-labelled DNA probes[26]. Total RNA isolated from stable transfected K562 cells carrying various LCR/β-globin gene episomal constructs was simultaneously probed for β-globin transgene and endogenous γ-globin mRNA. The β-globin gene signals were quantified and normalised using the endogenous γ-globin mRNA signal as an internal reference (see Materials and methods).

The β-globin gene alone gave very low levels of expression that were barely detectable in our assay (Figure 2A, lanes 1 and 2). The addition of βLCR HS2 (Figure 2A, lanes 3 and 4) and HS3 (Figure 2A, lanes 5 and 6) alone, stimulated expression approximately 10-fold on an episome copy number basis (Figure 2B, columns 2β and 3β). In contrast, βLCR HS4 (Figure 2A, lanes 7 and 8) gave little (2-fold) transcriptional enhancement (Figure 2B, column 4β). Constructs containing combinations of two of the βLCR HS sites gave varying levels of β-globin expression. HS3 and 4 (Figure 2A, lanes 13-14) gave levels of expression only

slightly greater than that obtained by HS3 or HS2 alone (Figure 2B, columns 43β). The combinations HS2/4 (Figure 2A, lanes 15-16) and HS2/3 (Figure 2A, lanes 11-12) induced a significant increase in transcription (average of 35-fold and 17-fold respectively; Figure 2B, columns 42β and 32β). The highest levels of expression (approximately 95-fold increase), were observed when the β-globin gene was under the control of all three of the HS sites tested (432β; Figure 2A, lanes 9-10; Figure 2B).

Expression of βLCR/β-globin gene episomal constructs in non-erythroid HeLa cells

In order to determine whether the βLCR HS sites maintain their ability to drive strict erythroid specific gene expression from within an REV context, a number of key constructs were analysed in non-erythroid HeLa cells. Expression of the human β-globin gene was determined by the same S1-nuclease protection assay that was used to analyse the transfected K562 cells. The ubiquitously expressed hnRNPA2 mRNA was used as internal reference to normalise the β-globin signal (see Materials and methods).

Although a signal of human β-globin mRNA could be detected from cells transfected with the β-globin gene alone, the addition of HS2, HS3 or the HS234 combination, failed to enhance expression (Figure 2C). These data demonstrate that the βLCR confers tissue-specific expression from within REVs.

Maintenance of episomal status and gene expression

In order to test the maintenance and stability of gene expression of the βLCR/β-globin/REVs within K562 cells as a function of time, stable transfected pools

containing the β-globin gene with βLCR HS2, HS3 and the HS234 combination were cultured continuously for 30 days in the absence as well as presence of hygromycin drug selective pressure. Southern blot analysis was used as before (Figure 1B) to assess episome copy number over the 30-day period of culture. The results showed that in the presence of hygromycin there was no significant drop of episomal copy number whereas in the absence of selection there was a gradual decrease (data not shown). This rate of episomal loss, however, varied between pools and appeared to largely depend upon the copy number at the initial stages of the transfection before removal of hygromycin from the culture medium.

Since the rate of episome loss was different for each pool, β-globin gene expression was determined at the point where 50% of the episomes were lost from the cultures. In the absence of hygromycin, the 432β construct maintained β-globin gene expression per copy at a similar (80-100%) level to that observed prior to commencement of the 30-day period of culture in the absence of the drug (Figure 3A, left hand panel; 432β columns). However, the 2β or 3β episomes both showed a significant decline in β-globin gene expression after hygromycin was removed from the cultures (Figure 3A, left hand panel). The pools with the 2β construct gave 10-20% of the original value of β-globin gene expression per episome copy (Figure 3A, left hand panel; 2β columns) whilst the 3β construct showed only 4-6% (Figure 3A, left hand panel; 3β columns). Surprisingly, similar results were obtained with the parallel cultures growing in the presence of hygromycin (Figure 3A, right hand panel). Despite no obvious drop in episomal copy number, β-globin gene expression markedly decreased over 30 days of culture in the pools harbouring either 2β (41-59%) or 3β (6.5%) when compared to the level seen on day 1 (Figure 3A, right hand

panel; columns 2β and 3β). In contrast, 432β maintained β-globin gene expression at the same level throughout the 30-day culture period (Figure 3A, right hand panel; columns 432β). FISH analysis confirmed that this resulted from episomal rather than integrated copies of the 432β construct (Figure 3B).

Discussion

Replicating episomal vectors based on viral origins of replication are showing increasing promise for use in gene therapy[1,6-8]. In addition to efficient delivery, advances in two areas need to be made in order for the utility of this system to be realised; episome maintenance and regulated gene expression. In this report we address the problem of obtaining strict tissue specific, long-term gene expression from within REVs. We demonstrate that the DNaseI HS sites that constitute the β-globin locus control region (βLCR) are able to confer cell-type specific gene expression from within REVs. HS sites 2,3 and 4 act synergistically to give an approximately 100-fold increase in expression compared to the human β-globin gene alone in erythroid K562 cells (Figure 2B). In contrast these elements are inactive in promoting enhanced transcription in non-erythroid HeLa cells (Figure 2C).

Previous studies have shown that only HS2 is able to function from within non-replicating plasmid constructs in experiments involving transient transfection. This classical enhancer activity localises to the NF-E2/AP-1 dimer binding site within this element and is capable of enhancing transcription in both erythroid and non-erythroid cells[27-29]. Interestingly, our results with REVs show that HS3 alone is able to increase transcription by about 10-fold over that obtained with the human β-globin gene alone

(Figure 2B). In general the performance of individual and combinations of βLCR HS sites in REVs reflects that seen in stable transfected tissue culture cells rather than transient transfection assays. As stable integrated transgenes in tissue culture cells, HS2[30,31] and HS3[30,32] but not HS1 or 4[30,33] are able to promote efficient erythroid specific gene expression. Combinations of HS sites act co-operatively to enhance transcription still further[30].

This difference in the activity of βLCR elements when assayed by transient transfection or within REVs, may be due to the fact that REVs adopt a well ordered nucleosomal configuration akin to that of native chromatin[17,18] Since most (HS1, 3 and 4) βLCR elements work only when integrated within the host cell genome, the chromatin configuration adopted by REVs would appear to provide the necessary environment for their function. In support of this idea is the report that HS2 can remodel chromatin on REVs[34] although this study addressed neither tissue-specificity nor the cumulative effect of combining βLCR HS sites.

The majority of studies addressing gene expression from within REVs, have focused on the use of ubiquitously acting classical promoter-enhancer combinations such as RSV[35] and CMV[11] The question of how to obtain efficient tissue-specific expression has not been thoroughly addressed. Inducible systems including the heavy metal responsive human metallothionein II_A promoter[35], the ionophore stimulated rat GRP78 promoters[35] and the incorporation of the dioxin response element[10] have also been tested with varying degrees of success. The data presented in this report indicate that tissue-specific gene expression from within REVs can be achieved with LCRs.

Our data also show that the constructs containing the βLCR elements tested do not contribute to the maintenance of episomes as they are lost from cells cultured in the absence of drug selective pressure (Figure 3A). EBV-based vectors disappear from dividing cells over a period of approximately two months without selection[36,37]. Our results showing the episomal loss in K562 cells within one to two months are, therefore, consistent with previous studies. Other vector systems with improved persistence inside cells have been reported. A hybrid BAC-HAEC vector containing the EBV *oriP* and EBNA-1, persisted 3 months in dividing human cells grown in the absence of selection[38] Also, an SV40-based episomal vector carrying an scafford/matrix attached region (S/MAR) from the human interferon β-gene was shown to be able to replicate and maintain in the CHO cells over more than 100 generations without selection pressure[39]. Combination of such improved systems with LCRs could be tested for stable and tissue-specific gene expression

While the retention of episomes within cells is clearly important, our work indicates that the maintenance of tissue-specific transgene expression from within REVs is also a crucial consideration. Expression of the β-globin gene from REVs with either βLCR HS2 or HS3 alone significantly declines over time in the presence or absence of drug selective pressure (Figure 3A). The silencing of β-globin gene expression in the presence of drug selection is intriguing as it implies that regions of the same episome can be repressed while others (the hygromycin resistance gene in this case), can remain active. Our findings imply that inclusion of certain elements to counteract the cell's selective gene silencing mechanism will be necessary for the use of episomal constructs in gene therapy and that combinations of LCR elements can efficiently fulfil this requirement.

Materials and methods

Cells and cell culture

K562[24] and HeLa cells were maintained in DMEM (Life Technologies Inc.) containing 10% foetal calf serum, 2mM L-glutamine, 50μg/ml penicillin and 50 units/ml streptomycin. Cells were cultured at 37°C in 5% CO_2. Transfection of these cell lines by electroporation was performed using a Bio-Rad Gene Pulsor at a setting of 0.25kV and 960μF, with 20-50μg of supercoiled DNA. Stable transformed cells were selected in the presence of 250μg/ml hygromycin B. K562 cells harbouring stable integrated transgenes were generated by linearising gene constructs at the unique *Pvu*I site (within the ampicillin resistance gene on the vector) prior to electroporation. In general, stable pools were analysed 3-4 weeks after transfection except for long-term culture experiments when approximately $1x10^7$ cells were divided into media with or without hygromycin and grown for up to a further 30 days.

Episomal plasmid constructs

A human β-globin gene extending from a 5' *Hpa*I site at -815 bp to an *Eco*RV site 1685 bp downstream of the poly(A)-addition site in the plasmid GSE1758[31] was removed as a 4.1 kb *Eco*RV fragment and inserted into a blunted *Sal*I site in the polylinker of p220.2 (Figure 1A), an Epstein-Barr virus based vector containing *oriP*, EBNA-1 and hygromycin resistance genes[9]. This cloning step brings a number of extra restriction sites (including a unique *Sal*I site) 5' of the β-globin gene (Figure 1A). The elements of the βLCR were isolated as a 1.5 kb *Kpn*I-*Bgl*II HS2 fragment, a 1.9 kb *Hind*III HS3 fragment and a 2.1 kb *BamH*I-*Xba*I HS4 fragment[30]. These

elements either alone or in combination were inserted by blunt-end ligation into the unique *SalI* site 5' of the β-globin gene (Figure 1A). Constructs which contain more than one HS site were joined such that their order reflected that found in the native β-globin locus on chromosome 11 (Figure 2A). These βLCR/β-globin gene episomal constructs were then stably introduced into human erythroid K562 and non-erythroid HeLa cells.

Isolation of low molecular weight DNA fractions

Low molecular weight DNA was prepared by the method of Hirt[25]. Briefly, cells were washed with PBS and subsequently incubated with lysis buffer (0.6% SDS, 10mM EDTA) for 15 min at room temperature. NaCl was then added to a concentration of 1.4M. After precipitation overnight at 4°C, genomic DNA and cell debris were pelleted by centrifugation at 10,000 rpm for 10 minutes in a Beckmen J21 high speed centrifuge. The supernatant was extracted with phenol:chloroform (1:1 v/v) and the DNA precipitated by adding 2.5 volume of ethanol and storage at -20°C. The precipitated DNA was collected by centrifugation and resuspended in TE (10mM Tris-HCl, 1mM EDTA, pH 7.5) buffer.

Analysis of extrachromosomally replicating episomes

Total DNA was prepared from cells as described[40], digested with *BamHI* and *HindIII*, and resolved by electrophoresis in a 0.7% agarose gel. These DNA samples were Southern blotted and hybridised with ^{32}P-labeled pBluescript (Stratagene) which detects the ampicillin resistance gene on the p220.2 vector backbone. Gel electrophoresis, blotting and hybridisation were carried out using standard procedures[41].

Quantification of human β-globin mRNA

Total RNA was extracted from K562 and HeLa cells by selective precipitation in the presence of 3M LiCl and 6M urea[40,42]. Analysis of human β-globin transgene expression in these samples was by an S1-nuclease protection assay using end-labelled, double stranded DNA probes[26]. The 5' human β-globin probe was a 525bp *Acc*I cDNA fragment that gives an S1-nuclease protected product of 154 nucleotides[43]. The 5' human γ-globin probe was an 1819bp *Hind*III-*Bam*HI genomic fragment giving rise to a 205 nucleotide S1-protected product from exon II. The probe for human hnRNPA2 was prepared as follows. A PCR fragment starting from 330 bp upstream of exon 9 to the 3' end of this exon[44] was cloned into the EcoRV site of pBluescript. The probe was prepared by PCR amplification using primers corresponding to a position 545 within pBluescript (5'-TGC TGC AAG GCG ATT AA-3') and the 3' end of hnRNPA2 exon 9 (5'-CTC CTC CAT AGT TGT CAT AAC C-3'). This generated a 627 bp probe that results in a 122 nucleotide S1-protected product. Products were resolved after S1-nuclease digestion on a 6% polyacrylamide gel in the presence of 8M urea, dried and quantified by PhosphorImager (Molecular Dynamics) analysis.

Southern blot analysis for episomal copy number determination

Total DNA was isolated from K562 and HeLa cells as described[40]. Samples were digested with *Eco*RI and resolved by electrophoresis on a 0.7% agarose gel. Southern blots of these gels were probed with a 920bp *Bam*HI-*Eco*RI fragment spanning the second intron of the human β-globin gene. This probe detects both the endogenous β-globin genes (5.5 kb fragment) and a fragment of various sizes from different

transfected constructs. The fragment sizes for each construct (see Figure 2A) are: 6.45kb (β); 7.95kb (2β); 8.35kb (3β); 8.55kb (4β); 11.9 kb (432β); 9.85kb (23β); 10.45kb (34β) and 10 05kb (24β). The intensities of the two bands corresponding to episomal and the endogenous β-globin genes were quantified by PhosphorImager and their ratio used to determine episome copy number taking into consideration that K562 cells are trisomy for chromosome 11[24] and therefore possess three copies of the β-globin locus.

DNA fluorescence in situ hybridisation

DNA FISH was carried out as described[45] with modifications K562 cells were washed in PBS, resuspended in hypotonic buffer (10 mM HEPES pH7.5, 30 mM glycerol, 1 mM $CaCl_2$, 0.8 mM $MgCl_2$) for 10 min. Cells were then washed in methanol/acetic acid (3:1) 3-4 times over 30 min. Slides with spread cells were air-dried, dehydrated through successive washes in 70%, 90% and 100% ethanol, prior to treatment with 100 μg/ml of RNase A at 37°C for 15 min. The slides were then rinsed in water, dehydrated by ethanol wash steps as before and air-dried before denaturing in 70% formamide, 2x SSC for 2 min at 70°C. The slides were then immediately immersed in ice-cold 70%, 90% and 100% ethanol (3 min each) and then air-dried. The probe used for hybridisation was the 38 kb β-globin minilocus[46] cloned into the SalI site of the EBV-based cosmid vector cos203 (p220.2 with cos sites; see Figure 1A) which simultaneously detects the endogenous β-globin loci and the episomal constructs. DNA (2 μg) was labelled with digoxygenin by nick-translation in a 25 μl reaction consisting of 0 5 M Tris-HCl pH 7.5, 0.1M MgSO4, 1 mM dithiothreitol, 500 μg/ml bovine serum albumin, 0.5 mM dNTPs (dATP, dCTP, dGTP), 0.7 nM digoxygenin-11-dUTP, 5

units DNA polymerase (Boeringer Mannheim) and 0.004 units DNase I (Sigma)) for 40 min at 15°C. Digoxygenin lebelled probes were dissolved in hybridisation mix (50% formamide, 2x SSC, 200 ng/μl of salmon sperm DNA, 5x Denhardt's solution, 1 mM EDTA, 50 mM sodium phosphate pH 7.0). Probe (20-50 ng) was denatured at 75°C for 5 min in 40 μl of hybridisation mix and allowed to anneal at 37°C with 20-fold excess of human Cot-1 DNA for 30 min before applying to the dried and denatured slides. Hybridisation was performed overnight. Slide washes and probe detection were carried out as described[45]

Acknowledgements

We thank Bill Sugden for the p220.2 and cos203 EBV-based vectors. The excellent technical support of Alexandra Aitken is gratefully acknowledged. This work was funded under the European Union Framework IV Biomed-2 (contract no. BMH4-CT96-1279) and Biotechnology (contract no. BIO4-CT96-0414) programmes.

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EXHIBIT C

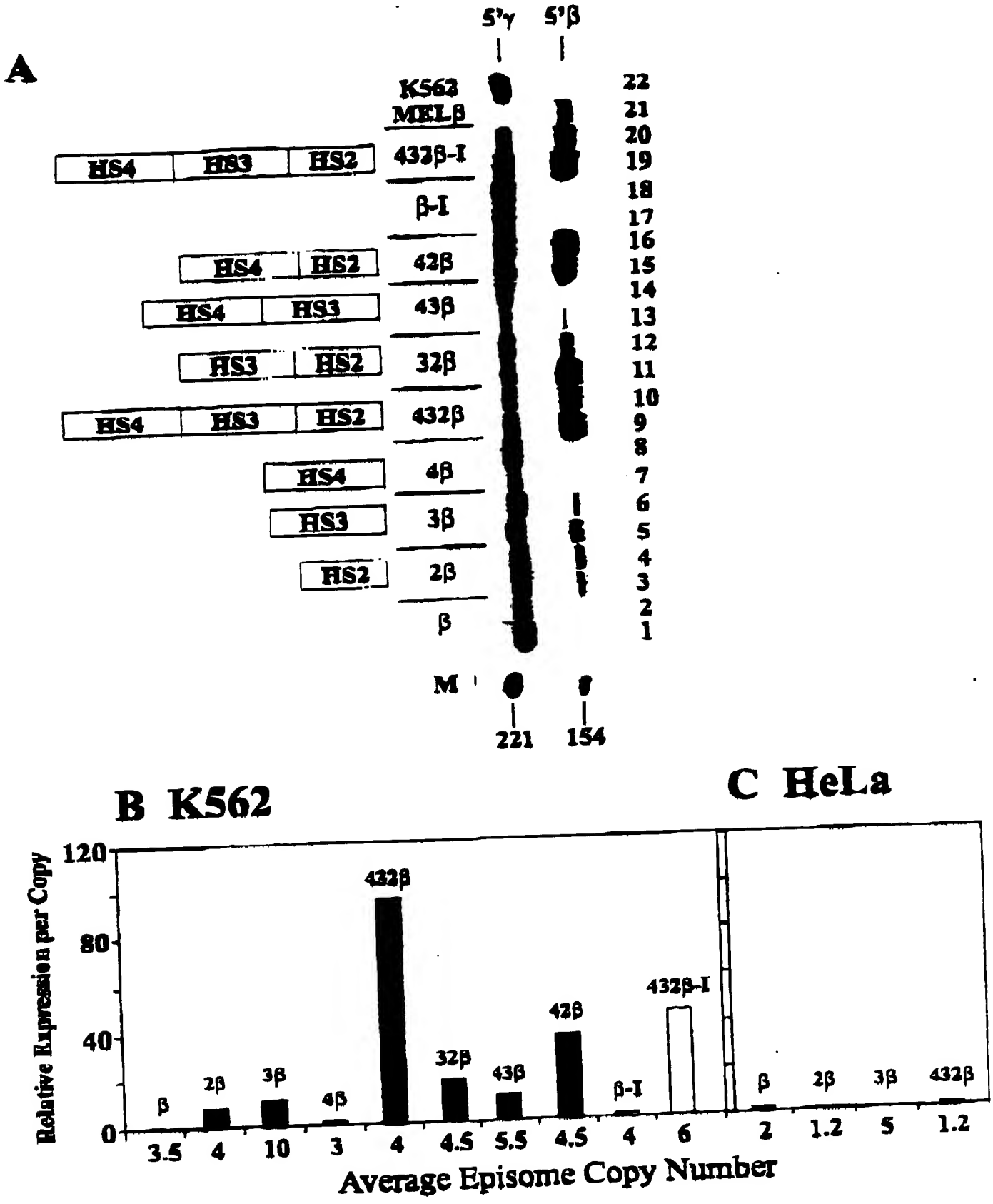
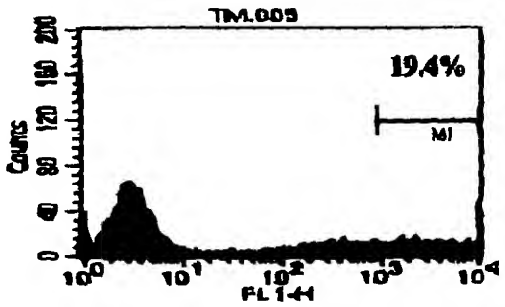


Figure 2: Chow et al., 2000

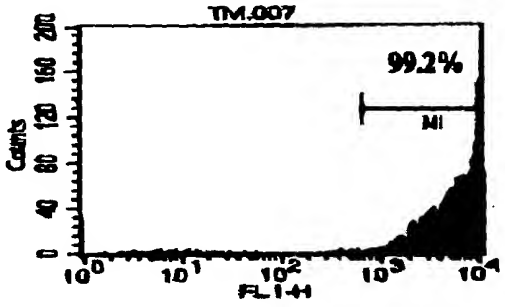
EXHIBIT D

EGFP expression in Jurkat cell lines on hygromycin selection, 76 days after transfection.

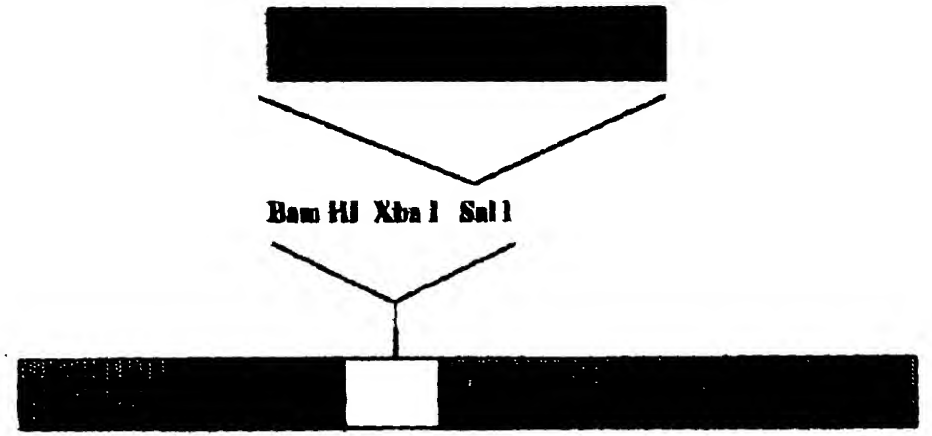
p220.EGFP



P220.CD2LCR



Maps of the vectors used



p220.CD2LCR

p220.EGFP